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Differential Metal Binding Interactions of the Imidazolinones Revealed by NMR and UV Spectroscopy

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Abstract: NMR and UV spectroscopy and molecular modeling methods were applied to probe the interaction of the two imidazolinones, imazethapyr (5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)nicotinic acid) and its structural isomer CL 303,135 (5-ethyl-3-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)picolinic acid), with metal ions. Both the imidazolinones inhibit the enzyme acetohydroxyacid synthase (AHAS) *in vitro*. However, while imazethapyr is a herbicide that is used widely in agriculture, CL303,135 does not exhibit herbicidal activity. Imazethapyr and CL303,135 exhibited considerable differences in their interactions with metals. In the metal complex of imazethapyr, the carboxyl moiety binds strongly and the pyridine nitrogen binds weakly with metals. In the case of CL303,135, both the pyridine nitrogen and the carboxyl group that are positioned *ortho* to each other participated strongly in the binding and were found to act together as a strong bidentate ligand to a metal ion. Both of the imidazolinones form predominantly 2 : 1 complexes with multivalent metal ions. However, imazethapyr binds two orders-of-magnitude more weakly ($1{\cdot}0 \times 10^{9}$ M^{-2}) with metal ions compared to CL303,135 ($1{\cdot}7 \times 10^{11}$ M^{-2}). The interactions of the model compounds, nicotinic acid and picolinic acid, with metals were examined similarly. It was concluded that the strong affinity of CL303,135 for metals compared to imazethapyr may affect its absorption from soil into plants, or its translocation in plants, thereby explaining the differences in herbicidal activity of imazethapyr and CL303,135.

Key words: CL303,135, imazethapyr, metal complex, molecular modeling, NMR, UV

1 INTRODUCTION

Imidazolinones such as imazapyr (2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)nicotinic acid), imazaquin (2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl) quinoline-3-carboxylic acid) and imazethapyr (5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)nicotinic acid) are all commercial herbicides used widely in agriculture.[1,2] They exhibit extremely low mammalian toxicity and high efficiency, resulting in very low application rates and low environmental impact.[3] They all inhibit the enzyme acetohydroxyacid synthase (AHAS; acetolactate synthase; acetolactate pyruvate-lyase (carboxylating); EC 4.1.3.18), which is present in plants and bacteria, but not in animals.[4] AHAS catalyzes reactions involving pyruvate and 2-ketobutyrate in the biosynthesis of the branched-chain amino acids leucine, isoleucine and valine. However, not all imidazolinones that are AHAS inhibitors are able to function as herbicides. For example, two isomers of an imidazolinone, CL303,135 and imazethapyr, exhibit comparable inhibition of AHAS *in vitro*, but on the other hand exhibit a considerable difference in herbicidal activity.[5] This is thought to arise because herbicidal activity also depends on rapid absorption and translocation of the inhibitor in the xylem and phloem,

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and accumulation in the meristemic regions, as well as on the rate of metabolism of the inhibitor. While the absorption, translocation and metabolism of imazethapyr in plants has been thoroughly studied as a requirement for the registration of the herbicide with government regulatory agencies, there is currently no comparable information for CL303,135 due to the high cost of such studies. However, such studies, when combined with the metal binding studies presented in this paper, could provide valuable mechanistic information.

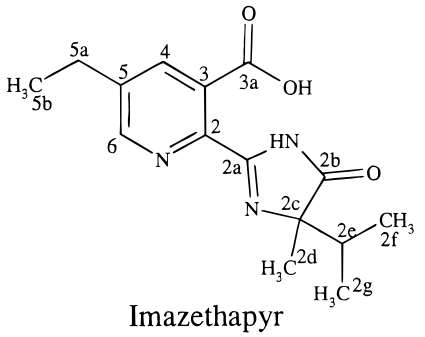
Multiple factors may affect the absorption and translocation of herbicides in plants. One hypothesis is that the strong binding of imidazolinones with metal ions that are present in soils and plants may affect their absorption and translocation, and even their interaction with AHAS. Copper is found commonly in many soils and plants. It has been argued by McBride[6] that organic molecules in soil act to mobilize or immobilize Cu^{2+}, with the net effect depending on the nature of the adsorbing mineral as well as the type of organic. Thus, if immobilization of an inhibitor by copper occurs in the soil, it will prevent uptake of the inhibitor by the plant. On the other hand, if the inhibitor is taken up by the plant, strong complexation by a metal may simply prevent effective translocation in the plant. Earlier studies of the interaction of imidazolinone herbicides with soil indicated that the herbicides imazethapyr and imazaquin showed a much higher affinity for copper-saturated montmorillonite clay than for calcium- or potassium-saturated clay (D. L. Shaner, unpublished results). Therefore, in the present work, copper(II) was used in comparative studies of interactions of metal ions with imidazolinones to explore why many AHAS inhibitors are not effective herbicides.

We selected two isomers of an imidazolinone, imazethapyr and CL303,135 (Fig. 1), that exhibit comparable *in-vitro* activity against AHAS (I_{50} 4 μM and 13 μM, respectively), and at the same time exhibit a considerable difference in their herbicidal activity (63 g ha^{-1} versus >4000 g ha^{-1}, respectively).[5] NMR and UV spectroscopic and molecular modeling methods were applied to study the interactions of the two imidazolinones with metal ions. NMR is a powerful method to study molecular interaction as it gives information at the atomic level on the distance of two interacting molecules. Both NMR and UV methods yield information on the binding affinity and composition of complexes formed between organic molecules and metals. Possible structures for the copper complexes of CL303,135 and imazethapyr are proposed using molecular modeling tools based on the NMR and UV binding data. The role of the imidazolinone ring in these compounds is also predicted by carrying out limited studies on their analogs, nicotinic and picolinic acids (Fig. 1), respectively. The inhibition kinetics of AHAS enzyme by the imidazolinones studied in presence of copper is also reported.

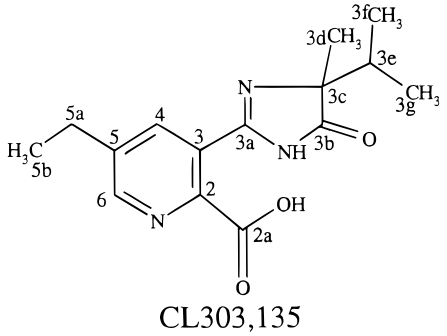
2 EXPERIMENTAL

2.1 Materials

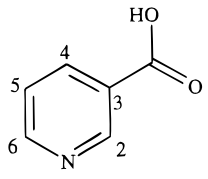
As the diamagnetic Cu(I) is sensitive to light and air and readily degrades to the Cu(II) state, Cu(II) was used for the binding studies. Copper(II) chloride and sulfate, nicotinic and picolinic acids were purchased from Aldrich and europium(III) chloride from Fluka. Imazethapyr and CL303,135 were obtained from the Analytical Standards Distribution at American Cyanamid Company. All the samples were dried under vacuum for



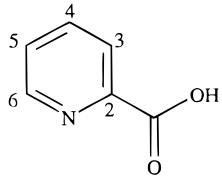
Imazethapyr



CL303,135



Nicotinic Acid



Picolinic Acid

Fig. 1. Structures of imidazolinones and their analogs: Imazethapyr, CL303,135, nicotinic acid and picolinic acid.

48 h before use. SigmaPlot software (Jandel Scientific, CA) was used for curve fit analysis of the binding data and for the linear regression analysis.

2.2 NMR Studies

The interactions of imazethapyr and CL303,135 with copper and europium were studied by [^{1}H] and [^{13}C]NMR under the same conditions of pH ($\sim$5–6) and concentration ($\sim$5 mg ml^{-1}) in deuterium oxide. The pH 5–6 was selected for these studies to maintain the carboxyl group mostly in the anionic state (CO_2^-) and avoid the precipitation of copper, and also because a small change in pH around this value due to the addition of metal does not affect the chemical shift of the imidazolinones significantly. [^{1}H] and [^{13}C]NMR spectra were obtained on Bruker AM-500 and AMX-300, and AMX-500 spectrometers. Sodium 3-trimethylsilylpropionate-2,2,3,3-d$_4$ (TSP-deuterated) was used as an internal chemical shift reference for deuterium oxide (D_2O) solvent. Typical conditions for [^{1}H]NMR were as follows: 16 K data points, 3–4 kHz sweep width, pulse width 3–5 μs (30°); relaxation delay 10 s. For [^{13}C]NMR: 32 K data points, 20–30 kHz sweep width, pulse width 3 μs (30°), relaxation delay 1–3 s. To assign carbon spectra of imidazolinones with and without metal, two-dimensional heteronuclear multiple bond correlation (HMBC) experiments[7–9] were carried out using a triple resonance gradient probe on the Bruker AMX-500 instrument. HMBC data were collected using 4–5 mg of imidazolinone in 0·75 ml D_2O (pH 5–6) at room temperature using 2028 × 512 data matrix with acquisition times of 80 and 200 ms in the F1 and F2 dimension, respectively. Forty scans were recorded per t_1 value and the total measuring time was approximately 15 h.

2.3 UV Studies

UV spectra were obtained on a Perkin-Elmer Lambda 4B UV/VIS spectrophotometer. Cells with a 1-cm path length were used. For studies of the complexes, solutions (3 ml) containing the imidazolinone (0·05 to 0·1 mg ml^{-1} in 0·05 M ammonium sulfate buffer, pH 5·5) were placed in the sample and reference cells. Copper sulfate solution (0·004 M, 10–200 μl) was added to the sample cell and an equal volume of buffer (without copper) to the reference cell, and the difference spectra were recorded.

2.4 pH Measurements

For the NMR studies, pH was measured using a special NMR pH electrode and a Corning pH meter, model 245. No correction was applied for the deuterium isotope effect of D_2O. Orion Research pH/millivolt meter 811 was used for the UV studies. The pH meter was standardized at a pH of 4·0 and 7·0 at 21°C. The pH was adjusted using dilute hydrochloric acid and sodium hydroxide to the accuracy of $\pm$0·1.

2.5 Molecular modeling

The structures of the imidazolinone–copper complexes were calculated using a suite of 'CAChe' molecular modeling programs on a Macintosh Quadra 900 computer equipped with an internal CAChe card. First, the geometries of the initial structures were optimized using augmented molecular mechanics with the MM2 force field parameters. The final geometry was found using ZINDO with INDO/1 (Intermediate Neglect of Differential Overlap) parameters. ZINDO is M. C. Zerner's INDO/1 semi-empirical molecular orbital calculation program using a valence-electron only procedure. The INDO/1 parameter set contained spectroscopic parameters for all of the elements of concern, including copper.

2.6 Enzyme extraction

AHAS was extracted from Black Mexican Sweet (BMS) corn cells. The growing conditions for BMS corn cells have been described previously.[10] Cells were powdered in liquid nitrogen and then homogenized in 100 mM potassium phosphate buffer (pH 7·0) containing pyruvate 10 mM, magnesium chloride 5 mM, EDTA 5 mM, flavin adenine dinucleotide (FAD) 100 mM, valine 1 mM, leucine 1 mM, glycerol 100 ml litre^{-1} and dithiothreitol 1 mM. The homogenate was filtered through a nylon cloth and centrifuged at 25 000*g* for 20 min. The supernatant was desalted on a Bio-Rad Econo-Pac 10 DG column (Bio-Rad, Richmond, CA) that had been pre-equilibrated with 100 mM potassium phosphate buffer (pH 7·0) containing sodium pyruvate 100 mM, magnesium chloride 10 mM, thiamine pyrophosphate 1 mM and FAD 10 mM. The desalted enzyme extract was used for the assay procedure.

2.7 AHAS assay

AHAS activity was measured by estimation of the conversion of pyruvate to acetolactate, after conversion of acetolactate to acetoin by decarboxylation in the presence of acid.[11] Standard reaction mixtures contained the enzyme in 50 mM potassium phosphate buffer (pH 7·0) containing pyruvate 100 mM, magnesium chloride 10 mM, thiamine pyrophosphate 1 mM and FAD 10 mM. This mixture was incubated at 37°C for 1 h. The

reaction was stopped by adding sulfuric acid to a final concentration of 0·85%, or by adding sodium hydroxide solution (4 M) to a final concentration of 0·67 M. The reaction product was allowed to decarboxylate at 60°C for 15 min. The acetoin formed was determined after incubating with creatine (0·17%) and 1-naphthol (1·7%) by the method of Westerfeld.[12]

3 RESULTS AND DISCUSSION

3.1 Interaction of imazethapyr and CL303,135 with copper by [^{1}H]NMR

In deuterium oxide at pH 5·5 the paramagnetic Cu(II) broadens the resonances for protons 6, 5a, 2d, 2e and 2f/2g of both the pyridine and imidazolinone rings of imazethapyr (Fig. 2), suggesting the participation of both rings in the interaction with copper. In the case of CL303,135, it is only the pyridine ring that participates in the binding with copper as only the proton resonances 6 and 5a of pyridine ring were broadened by copper (Fig. 3). Similar results were obtained for comparable studies carried out in acetonitrile (data not shown).

The extent of line broadening caused by copper can be related to the distance between copper and the specific proton. The Solomon and Bloembergen

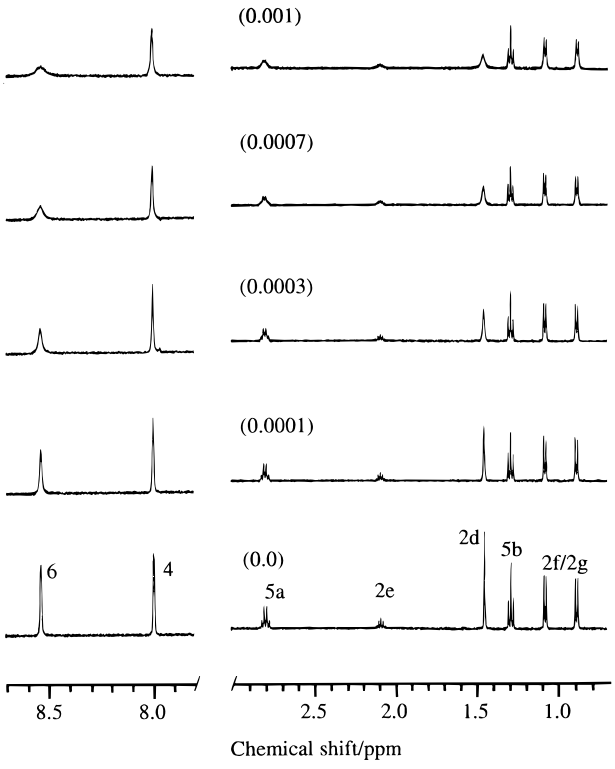


Fig. 2. [^{1}H]NMR spectra of imazethapyr ($1{\cdot}61 \times 10^{-5}$ M) and its complex at different mole ratio (f) of copper to imazethapyr (given in parenthesis) in deuterium oxide at pH 5·0.

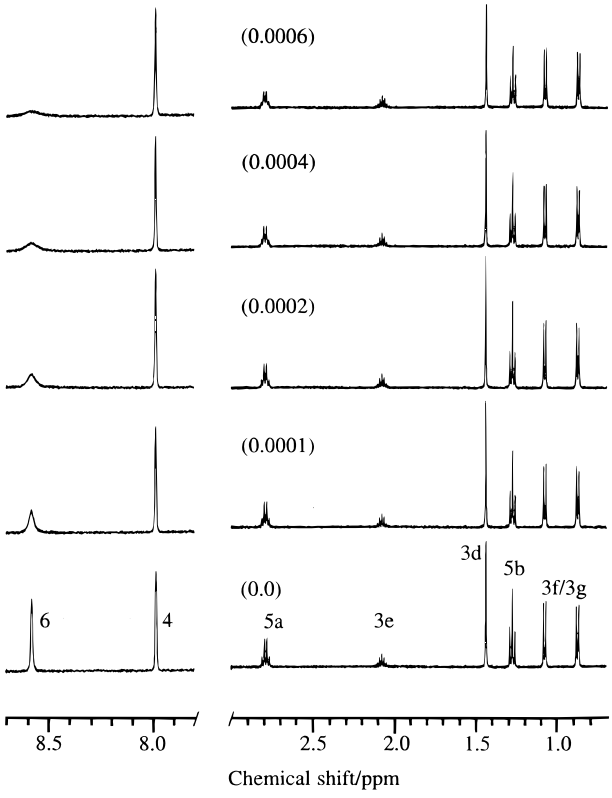


Fig. 3. [^{1}H]NMR spectra of CL303,135 ($1{\cdot}48 \times 10^{-5}$ M) at different mole ratio (f) of copper to CL303,135 (given in parenthesis) in deuterium oxide at pH 4·8.

equation[13,14] for the electronic contribution to the spin-spin relaxation time, T_2, can be related to the observed linewidth and simplified to:

$$\frac{1}{T_{2p}} = \frac{1}{T_{2(\text{obs})}} - \frac{1}{T_{2(0)}} = \Gamma \frac{\mathbf{f}}{\mathbf{r}^6} \tag{1}$$

This equation is based on assumptions such as the extreme narrowing condition, negligible scalar contribution and fast chemical exchange.[15] $T_{2(\text{obs})}$ and $T_{2(0)}$ are the observed relaxation times in the presence and absence of paramagnetic ion, respectively. The transverse relaxation times, T_2, were estimated from the linewidths ($\Delta\nu$) at half peak height ($T_2^{-1} = \pi\Delta\nu$). **f** is the ratio of paramagnetic copper ion concentration to imidazolinone concentration, **r** is the metal ion-proton internuclear distance, and Γ is a constant. Qualitatively, the above equation means that the protons closest to the copper ion will be preferentially broadened and this is highly sensitive because of the sixth-power dependence. Distances can be compared quantitatively by taking the slopes for two protons from the plots of T_{2p}^{-1} against **f** (Fig. 4). Such NMR-derived relative distance information is used to determine the site(s) of the interaction and also to model the structure of the complex using standard molecular modeling methods (see below). In the part (b) of Fig. 4, the slope of the curve for the proton 6 is greater than that of the protons 5a, indicat-

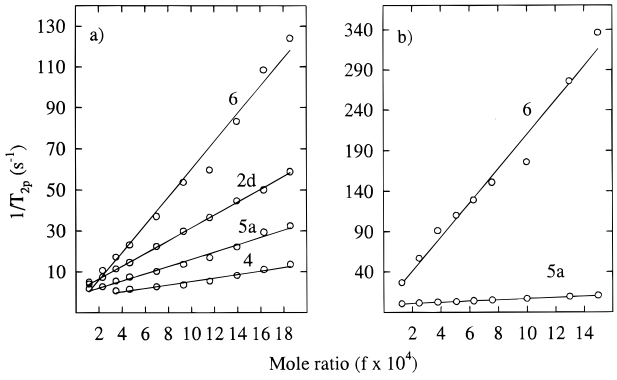


Fig. 4. Plot of the measured values of $1/T_{2p}$ for the protons of (a) imazethapyr and (b) CL303,135 as a function of the mole ratio (f) of copper to imazethapyr and CL303,135, respectively. The solid line represents the fitted first-order linear regression line. In (a), the slopes of the curves for the protons 6, 2d, 5a and 4 are 6·76, 3·12, 1·76 and 0·84, respectively. In (b), the slopes of the curves for the protons 6 and 5a are 21·16 and 0·75, respectively.

ing that the proton 6 is closer to copper than are the 5a methylene protons in the copper complex of CL303,135. Similarly, in the case of the imazethapyr complex, the slope for the proton 6 is greater than that of 2d, 4 and 5a protons (part (a) of Fig. 4), indicating that the proton 6 is again closer to copper than are protons 2d, 4 and 5a. The finding that the proton 6 is closer to copper than any other protons in the complexes of imazethapyr and CL303,135 indicates that the pyridine ring participates in the binding in both cases.

3.2 Binding of imazethapyr and CL303,135 with europium by [^{1}H]NMR

Copper was not useful for determining the composition and binding constant of the complex by NMR because it caused severe NMR line-broadening upon addition of far less than one equivalent (Figs 2 and 3). Therefore, one of the lanthanides, europium, was used in additional NMR studies to determine the composition and binding affinity of the complex.

Lanthanides are widely used to study the structure and conformation of small organic molecules, peptides and proteins by NMR.[16–18] When the lanthanide cation is bound to a ligand, it can induce changes in both chemical shift and nuclear spin relaxation. Lanthanides can be classified into three groups: (i) cations such as Eu^{3+} and Nd^{3+}, which have very short electron relaxation times, causing chemical shift perturbation but negligible line broadening; (ii) cations such as Gd^{3+} and Eu^{2+}, which have long electron relaxation times, causing only a large isotropic broadening effect; (iii) cations such as Ho^{3+} with intermediate relaxation times that cause changes in both line width and chemical shift. For the studies of imidazolinone complexes, Eu^{3+} was used. Both Cu^{2+} and Eu^{3+} are paramagnetic cations. Both can form four- and six-coordinate complexes. Unlike d-block transition metals like copper, the lanthanides, for which 4f electrons are responsible for their properties, can also form higher than six-coordinate complexes.[19] The NMR data obtained for the lanthanide complexes are easier to interpret than NMR data for transition metals. The 4f electrons in lanthanides are well screened and their orbitals have little overlap with those of ligand electrons. Therefore, all perturbations by lanthanides can be attributed to the pseudocontact (though-space) interaction, and the contact (though-bond) interaction may be safely neglected.

Eu^{3+} affects only NMR chemical shifts and does not broaden the lines significantly. Therefore, stoichiometric amounts of europium can be added. In the europium binding studies, the Eu^{3+} was titrated against the imidazolinone by following the changes in the chemical shift of the proton NMR resonances. In the case of CL303,135, large chemical shift changes were observed for proton 6 upon the addition of europium, and a maximum was reached with the addition of two equivalents (part (b) of Fig. 5). In contrast, a relatively small chemical shift change was observed for proton 6 of imazethapyr for the same amount of europium, and it continued to increase asymptotically even after the addition of four equivalents of europium (part (a) of Fig. 5). Qualitatively, these observations reveal that the binding affinity of CL303,135 for europium is higher than that of imazethapyr.

A non-linear least-squares curve fitting routine in the SigmaPlot scientific graphing software was used to analyze the binding data for a simple 1 : 1 complex according to the equilibrium:

$$M + L \leftrightarrow ML \qquad (2)$$

where M is a metal and L is an imidazolinone. According to Stockton and Martin,[20] the observed population

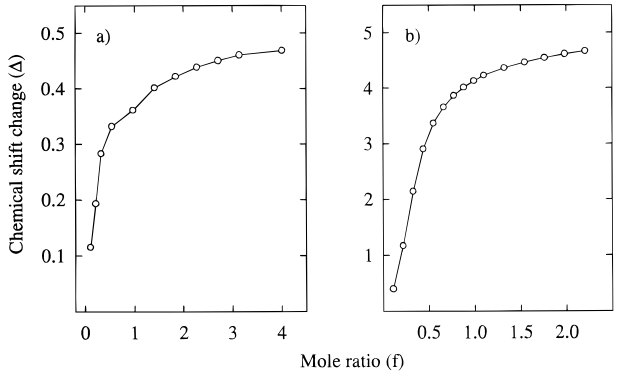


Fig. 5. Plot of the measured NMR chemical shift changes (Δ) for the proton 6 of (a) imazethapyr (0·027 M) and (b) CL303,135 (0·026 M) as a function of the mole ratio (f) of europium to imazethapyr and CL303,135, respectively.

average shielding, σ, is given by

$$\sigma = [p_f \sigma_f + p_c \sigma_c],$$

where p_f and p_c are the populations, and σ_f and σ_c the NMR shieldings, of the free and complexed ligand, respectively. The shifts Δ and Δ_c are then defined as $\Delta = \sigma - \sigma_f$ and $\Delta_c = \sigma_c - \sigma_f$. Thus, the NMR shift Δ can be related to the concentration of the complex $[ML]$ by the formula:

$$\Delta = \frac{[ML]}{L_0} \Delta_c \tag{3}$$

where L_0 is the total amount of ligand (free + bound).

In the titration of imidazolinones against metal, the metal ion concentration, $[M]$, is varied while the total imidazolinone concentration, L_0, remains constant. For every $[M]$, the corresponding Δ value was measured. The Δ value was also calculated theoretically by estimating the value of $[ML]$ as shown below from the metal and imidazolinone concentrations and assumed equilibrium constant K and Δ_c values.

$$[ML] = \frac{(L_0 + M_0 + 1/\mathrm{K}) - \sqrt{(L_0 + M_0 + 1/\mathrm{K})^2 - 4L_0 M_0}}{2} \tag{4}$$

The NMR binding data for both imazethapyr and CL303,135 do not fit the equation for a simple 1 : 1 complex (ML).

Since the NMR binding data do not fit the 1 : 1 complex equation, Job's method, also known as the continuous variations method,[21] was used to determine the stoichiometry of the complex. The overall concentration of the two species, [metal] + [imidazolinone], was kept constant and the mole fraction x = [metal]/([metal] + [imidazolinone]) was varied from 0 to 1. The quantity Δ multiplied by [imidazolinone] was plotted against x. Δ is the difference between the chemical shift of free imidazolinone and the observed value for a given mole fraction x. For both imazethapyr and CL303,135, the maximum of the plot occurred around 0·3 (Fig. 6), indicating the predominant existence of a 2 : 1 (imidazolinone : metal) complex.

The only one experimentally observed value of chemical shift, which is also the average of both the free and bound imidazolinone, and many unknown parameters preclude the curve-fit analysis of the NMR binding data for the 2 : 1 complex. Therefore, the reverse titration procedure[22] was used in which metal concentration (M_0) was kept constant and at least 10 times lower than the imidazolinone concentration (L_0), thus guaranteeing $L_0 \gg ML_2$. The imidazolinone concentration was varied. Under this reverse condition, the following

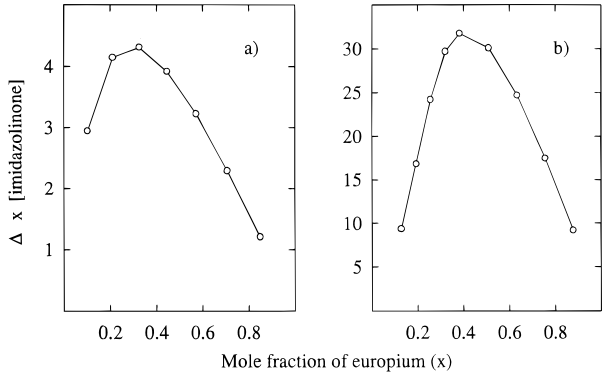


Fig. 6. Job's plot of the quantity $\Delta \times$ [imidazolinone] versus mole fraction (x) for the interaction of (a) imazethapyr and (b) CL303,135 with europium.

equation for binding constant K was obtained for the complex, ML_2.

$$L_0^2 = M_0 \Delta_c (L_0/\Delta) - 1/\mathrm{K} \tag{5}$$

Δ is the induced chemical shift change for a given concentration (L_0) and Δ_c is the chemical shift change for the complex ML_2. The intercept of the plot of L_0^2 versus L_0/Δ (Fig. 7) gives the binding constant. The binding constant for imazethapyr ($\mathrm{K} = 1{\cdot}5 \times 10^5$ м$^{-2}$) was found to be much less than that of the CL303,135 ($\mathrm{K} = 7{\cdot}4 \times 10^7$ м$^{-2}$).

3.3 Interaction of imazethapyr and CL303,135 with europium by [^{13}C]NMR

As the [^{1}H]NMR data alone cannot be used to confirm the involvement of the carboxyl in binding, [^{13}C]NMR data were also collected. In [^{13}C]NMR studies, copper has not affected the chemical shifts of the ^{13}C resonances but caused severe line-broadening. Therefore, europium was used again as it affects predominantly

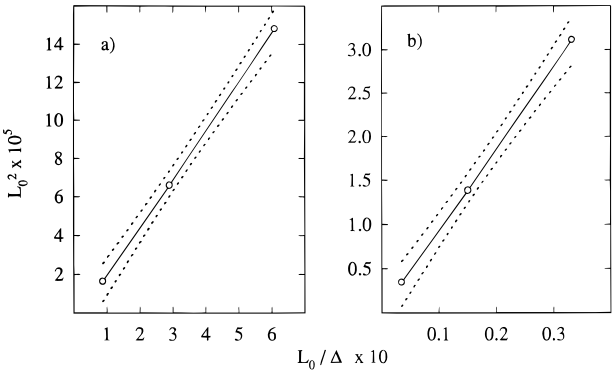


Fig. 7. The plot of L_0^2 versus L_0/Δ for the interaction of (a) imazethapyr and (b) CL303,135 with europium. The two dotted lines around the solid first-order regression line describe the 95% confidence intervals. The intercepts are −0·655 and −0·001 36 for (a) and (b), respectively.

NMR chemical shifts with little or no broadening of the lines.

For [^{13}C]NMR studies of interaction of imidazolinones with europium, one equivalent of metal for imazethapyr and a half-equivalent for CL303,135 was added and pH was maintained around 5–6. When an equivalent amount of europium was added to CL303,135, a few carbons were not detectable because of severe line-broadening, perhaps due to their strong interaction with europium. The [^{13}C]NMR spectra of imidazolinones and their complexes with europium were assigned using a carbon–proton multiple bond correlation experiment (HMBC). In the HMBC experiment, less sensitive nuclei carbons are detected indirectly *via* the more sensitive nuclei protons which are two or three bonds away from the connected carbons. The experiments with direct observation of carbon signals are not useful for assigning the [^{13}C]NMR spectra of imidazolinones and their complexes because of their low concentration, which is due to their poor aqueous solubility. The HMBC results including the assignments are listed in Table 1 for imazethapyr and CL303,135.

The quaternary carbons, 3 and 2a, that do not have any long-range correlation with protons were assigned based on comparison with similar compounds and chemical shift prediction. When the metal is bonded to the carboxyl oxygen, the carboxyl carbon is deshielded and shifts downfield (Table 1). At the same time, the carbon attached to carboxyl group is predicted to be shielded and, therefore, expected to be shifted upfield. Molecular orbital theory[23] predicts this charge transfer to be propagated along the carbon backbone, producing alternating shifts which decrease with the inverse third power of the distance. For imazethapyr, it was found that the carbon of the carboxyl group shifted downfield by 12 ppm and the carbon attached to the carboxyl group shifted upfield by 16 ppm (Table 1). All other pyridine ring carbons were affected to only a

TABLE 1
[^{13}C] Chemical Shifts for Imazethapyr and CL303,135 with and without Europium

	Imazethapyr		*Imazethapyr + Europium (1 : 1)*	
Carbon	*Chemical Shift* (δ)	*HMBC Results* (*Protons*)	*Chemical Shift* (δ)	*HMBC Results* (*Protons*)
2b	196·9		197·3	2d
3a	**176·1**	4	**188·7**	4
2a[a]	171·0		172·0	
6	151·9	4, 5a	152·9	4, 5a
5	146·5	6, 5a	147·1	6, 5a, 5b
2	144·3	4, 6	143·6	4, 6
4	139·4	6, 5a	140·7	6
3[a]	**138·3**		**122·0**	
2c	76·8	2d, 2f/2g	76·3	2d, 2f/2g
2e	37·2	2d, 2f/2g	37·7	2d, 2f/2g
5a	28·3	4, 6	28·7	4, 6, 5b
2d	22·6		23·0	2e
2f/2g	19·2, 19·0		19·5, 19·4	2e
5b	17·0	5a	17·3	5a
	CL303,135		*CL303,135 + Europium (1 : 0·5)*	
3b	197·1	3e	195·6	3d
2a[a]	**174·9**		**217·6**	
3a	**172·7**	4	**165·3**	
6	**153·4**	4	**167·5**	4, 5a
5	**144·2**	5b, 5a	**130·2**	5a, 5b
2	***154·5***	4	**139·2**	4
4	**140·5**	5a	**152·0**	5a
3[a]	***125·8***		**97·0**	
3c	76·5	3d, 3e, 3f/3g	76·3	3d, 3f/3g
3e	37·2	3d, 3f/3g	36·7	3d, 3f/3g
5a	28·1	4, 5b	29·1	5b, 4
3d	22·6	3e	21·8	
3f/3g	19·1, 19·0	3e	19·1, 19·0	3e
5b	17·1	5a	17·9	5b

[a] See text for assignment details.

small extent (around 1 ppm), and the imidazolinone ring carbons remained unaffected (Table 1), suggesting the strong participation of the carboxyl group. A weak participation of the pyridine nitrogen in the binding is also possible. However, in the case of CL303,135, the carbons of the pyridine ring and the carboxyl were affected strongly by the europium addition (Table 1), indicating the strong participation of the carboxyl group and the nitrogen of the pyridine ring in binding. Again, the carbons of the imidazolinone ring remained unaffected except the carbon 3a. The change in chemical shift of 3a could be due to charge transfer effect because of the binding of europium to the carboxyl and nitrogen of the pyridine ring. This is in agreement with the [^{1}H]NMR results that none of the protons in the imidazolinone ring was affected by the addition of copper.

From the [^{13}C]NMR experiments, it becomes clear that only the pyridine ring and the carboxyl are involved in binding for both the imazethapyr and CL303,135. This is in agreement with [^{1}H]NMR results for CL303,135 only. In the case of imazethapyr, it was found in [^{1}H]NMR studies that 2d and 2e protons of the imidazolinone ring were broadened by the addition of copper. This could still be possible without the direct involvement of the imidazolinone ring in the binding if the geometry of the copper complex is such that copper is close to those protons of the imidazolinone ring (see Section 4).

3.4 Binding of nicotinic and picolinic acids with europium by [^{1}H]NMR

Similar titration studies with europium were carried out for nicotinic and picolinic acids. These reference compounds lack the imidazolinone ring and serve as analogs of imazethapyr and CL303,135, respectively. These model compounds were chosen to avoid the possible binding sites from the imidazolinone ring. Nicotinic and picolinic acids showed a similar difference in the interaction behavior with europium to that of imazethapyr and CL303,135, respectively. The picolinic acid protons showed a large chemical shift change and reached a maximum change within the addition of one equivalent of europium (part (b) of Fig. 8) similar to CL303,135 (part (b) of Fig. 5). The chemical shifts of nicotinic acid protons changed gradually in small increments and kept changing asymptotically even after the addition of two equivalents of europium (part (a) of Fig. 8) similar to imazethapyr (part (a) of Fig. 5). This type of interaction behavior with europium indicates qualitatively that the nicotinic acid binds weakly with europium compared to picolinic acid.

As found with imazethapyr and CL303,135, the NMR titration data of europium against nicotinic and picolinic acids also do not fit a simple 1 : 1 complex equation, suggesting similar mechanisms of interaction to

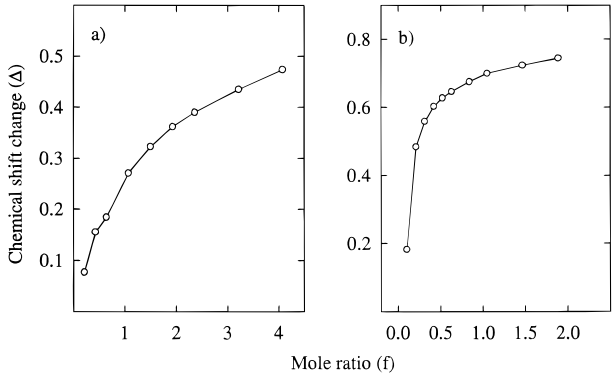


Fig. 8. Plot of the measured NMR chemical shift changes (Δ) for (a) proton 2 of nicotinic acid (0·025 M) and (b) proton 6 of picolinic acid (0·025 M) as a function of the mole ratio (f) of europium to nicotinic acid and picolinic acid, respectively.

those of imazethapyr and CL303,135, respectively. Perhaps the imidazolinone ring of the imidazolinones does not interact directly with the metal ion, which is in agreement with the [^{13}C]NMR findings. When the same reverse titration procedure was applied to nicotinic and picolinic acids, no measurable chemical shift change was observed for nicotinic acid because of a very weak complex formation. In the case of picolinic acid, the binding constant K was estimated to be $1{\cdot}0 \times 10^4$ M^{-2}, which is 1000 times less than that of CL303,135. Therefore, it can be concluded that the imidazolinone ring in imazethapyr and CL303,135 improves the binding affinity for metal but does not participate directly in the binding.

3.5 Binding copper to imazethapyr and CL303,135 probed by UV spectroscopy

As an alternative method, UV spectroscopy was applied, not only to check the NMR results but also because it permitted the use of copper directly in the binding studies. UV titration experiments were carried out, varying the copper concentration and keeping the imidazolinone concentration constant. A single isobestic point around 200 nm was observed for the imazethapyr complex (part (a) of Fig. 9), whereas two isobestic points around 200 and 270 nm were found for the CL303,135 complex (part (b) of Fig. 9). At higher concentrations of copper sulfate, a few spectra do not intersect at 200 nm (part (b) of Fig. 9), which could be due to absorption of copper sulfate itself. Since the copper solution absorbs at lower wavelengths, 200–250 nm, a wavelength around 290–300 nm was chosen for complexation studies. As found for the NMR binding data, the UV binding data also do not fit the simple 1 : 1 complex equation.

A modified Job's plot[24] was used to extract the binding constant in addition to the stoichiometry of the complex. The modified Job's plot uses a normalized *y*-scale instead of the absorbance scale. This *y*-scale is

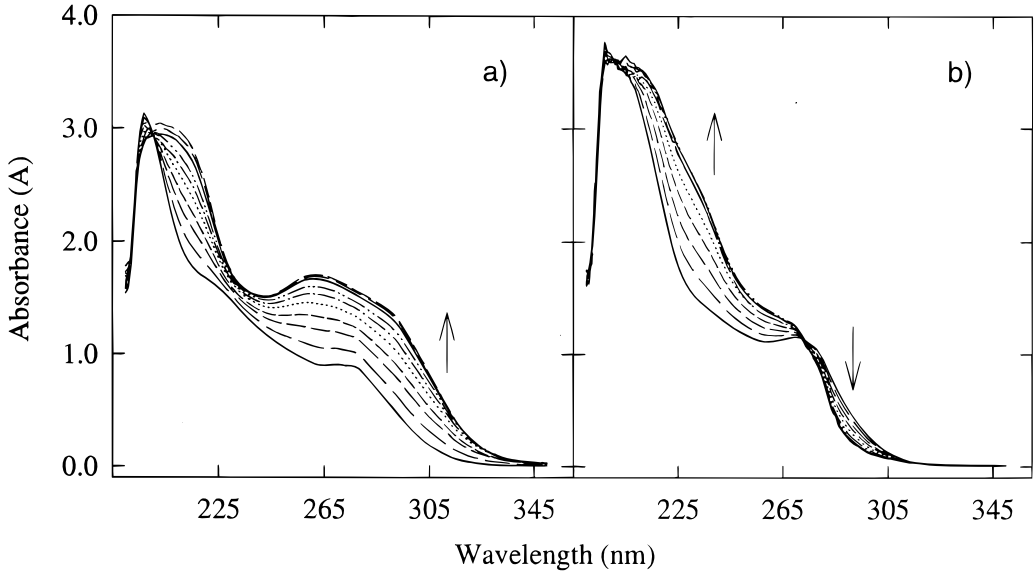


Fig. 9. UV spectra of the complexes of (a) imazethapyr ($1{\cdot}35 \times 10^{-4}$ M) and (b) CL303,135 ($1{\cdot}72 \times 10^{-4}$ M) with copper at various concentrations of Cu^{2+} solution ranging from $1{\cdot}37 \times 10^{-5}$ M to $1{\cdot}59 \times 10^{-4}$ M. The arrow indicates the direction of increasing concentration of Cu^{2+}.

based on the quotients obtained when the experimental absorbances (A) are divided by the maximum absorbance, A_{max}. The latter is obtained by measuring the absorbance of a solution containing an excess of imidazolinone over the metal concentration (x_{max}) against a reference solution containing an identical imidazolinone concentration. X_{max} is the mole fraction of metal of the stoichiometric composition of the complex. Using this normalization concept, the following equation was derived for the binding constant of the complex $M_m L_n$:

$$K = [(m + n)/T]^{(m+n-1)} m^{-m} n^{-n} y_{max} (1 - y_{max})^{-(m+n)} \quad (6)$$

where T is the total concentration of the metal and imidazolinone. In the modified Job's plot of y versus mole fraction of metal (x), the x_{max} occurred around 0·3 for both imazethapyr and CL303,135 (Fig. 10), indicating the predominant existence of a 2 : 1 complex. In the case of CL303,135, the plot is almost a perfect triangle, indicating very strong complex formation. For imazethapyr, the plot is slightly curved, indicating weaker complex formation than that of CL303,135.

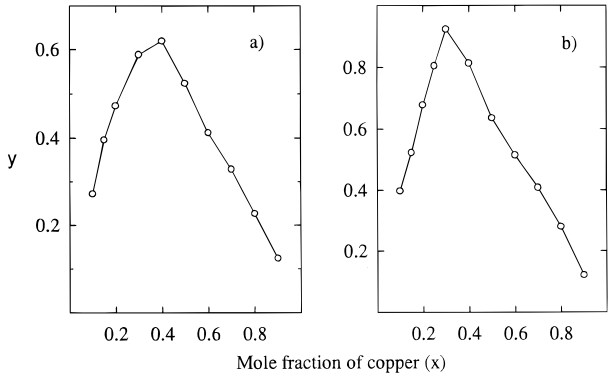


Fig. 10. Modified Job's plot of UV data of y against mole fraction (x) for the interaction of (a) imazethapyr and (b) CL303,135 with copper.

For m : n = 1 : 2, the above binding constant equation simplifies to:

$$\log K = 0{\cdot}3522 - 2 \log T + \log y_{max} - 3 \log(1 - y_{max}) \quad (7)$$

Using this binding constant equation and the y_{max} value from the plots (Fig. 10), the binding constants were estimated.

The binding constant for the CL303,135-copper complex ($1{\cdot}7 \times 10^{11}$ M^{-2}) is two orders of magnitude greater than that of the imazethapyr–copper complex ($1{\cdot}0 \times 10^{9}$ M^{-2}). This conclusion is consistent with the results from earlier NMR studies of europium binding, though the binding constants were lower ($\sim 10^5$ to $\sim 10^7$). The similar results obtained using europium justify the use of this lanthanide as a surrogate for copper in the NMR studies.

4 MOLECULAR MODELING

NMR and UV spectroscopy data indicated that CL303,135 forms a predominantly 2 : 1 complex with copper and only the pyridine ring nitrogen and the carboxyl group of CL303,135 are involved in the binding. Based on these data, only one possible structure for the copper complex of CL303,135 can be proposed, as shown in Fig. 11. The geometry was first optimized by a molecular mechanics energy minimization (augmented MM2), followed by a semi-empirical molecular orbital calculation using INDO/1 parameters optimized for

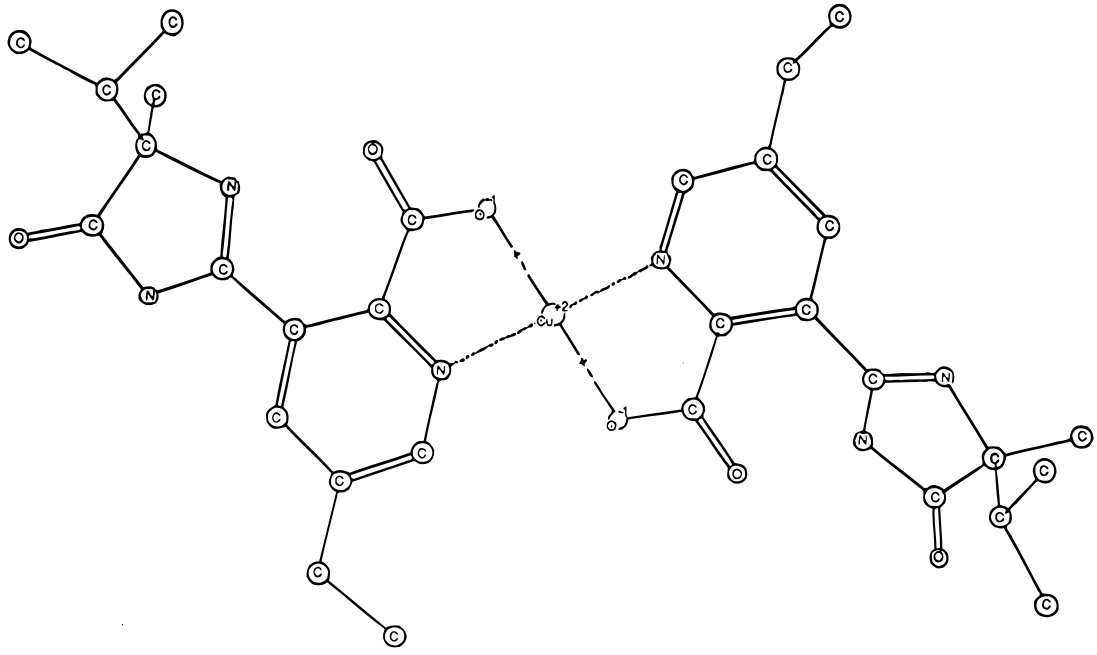


Fig. 11. A proposed structure for a 2 : 1 complex of CL303,135 with copper, involving only the pyridine ring.

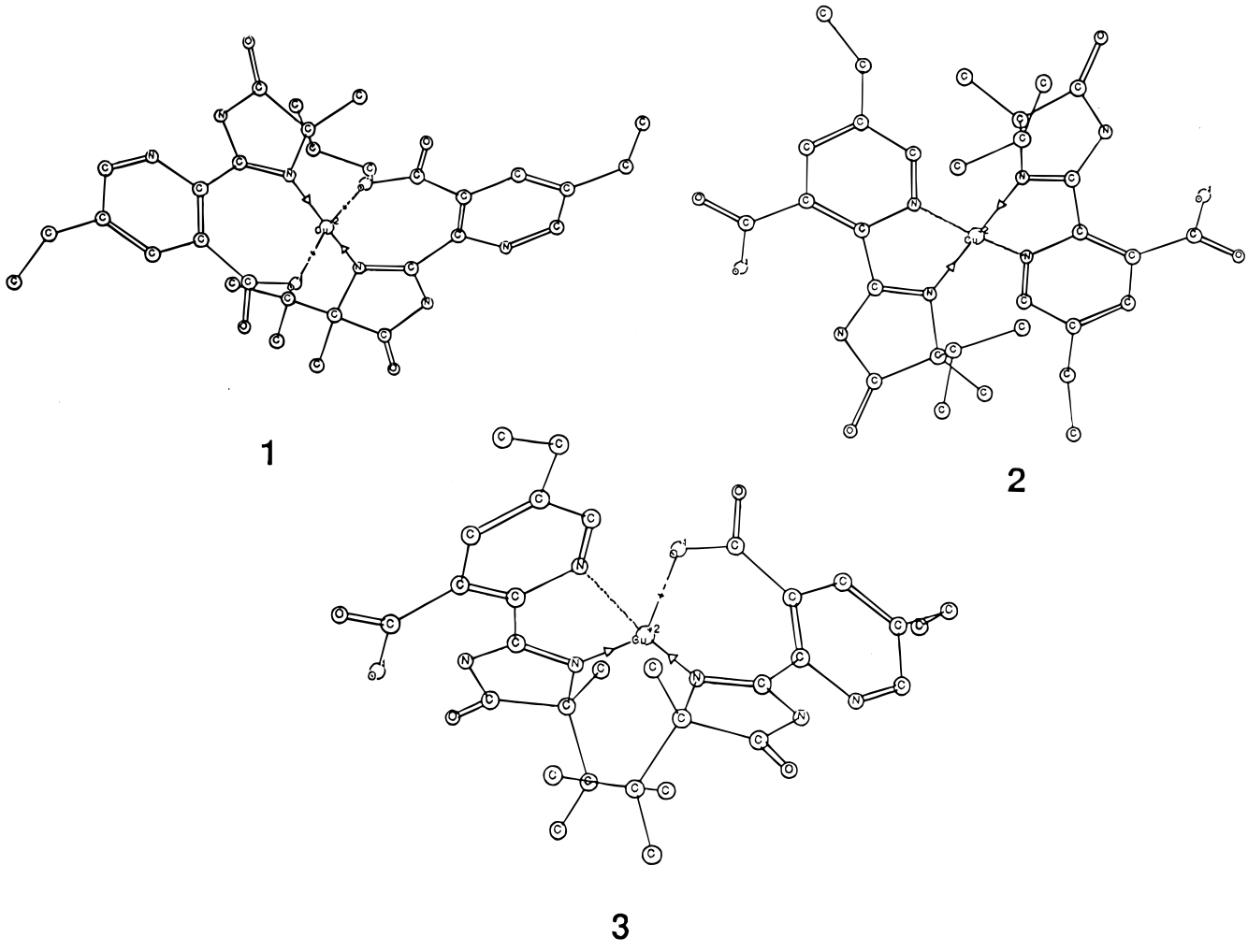


Fig. 12. Three possible structures for a 2 : 1 complex of imazethapyr with copper, involving both pyridine and imidazolinone rings.

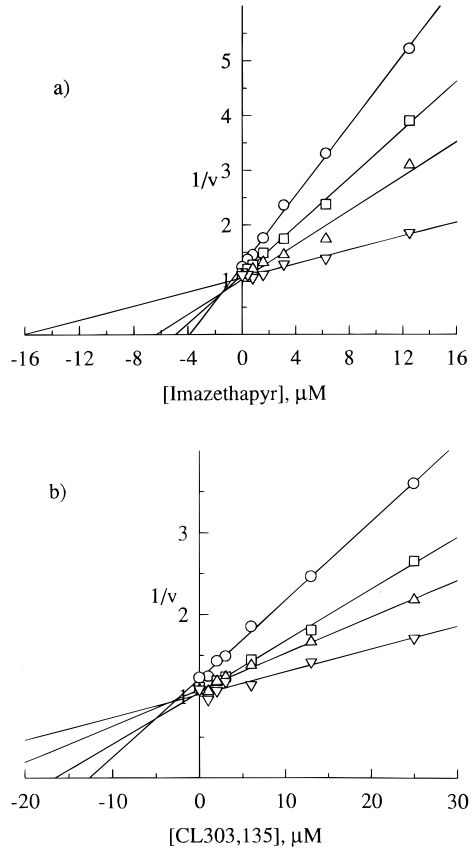


Fig. 13. Inhibition of AHAS by (a) imazethapyr and (b) CL303,135 at varying concentrations of cupric chloride solution: (▽) 0, (△) 6·25, (□) 12·5 and (○) 25·0 μM.

spectroscopy (ZINDO). The proposed structure satisfies the NMR data including the relative distances (see part (b) of Fig. 4), i.e. proton 6 is closer to copper than the 5a methylene protons. None of the imidazolinone ring protons is closer to copper in the model structure, as indicated by [^{13}C]NMR data, suggesting that the imidazolinone ring of CL303,135 is not involved in metal binding.

In the case of imazethapyr, the imidazolinone ring is positioned between the carboxyl group and the nitrogen of the pyridine ring. In [^{1}H]NMR studies with copper, the methyl group of the imidazolinone ring showed a significant broadening. If the methyl broadening is due to direct involvement of the imidazolinone ring in the binding, at least three models can be proposed for the 2 : 1 complex of imazethapyr with copper (Fig. 12): (1) The carboxyl group and the imine nitrogen of the imidazolinone coordinated to copper; (2) The nitrogen of the pyridine ring and the imine nitrogen of the imidazolinone ring coordinated to copper; (3) The nitrogen of the pyridine and the imine nitrogen of the imidazolinone ring from one molecule and the carboxyl group and the imine nitrogen of the imidazolinone ring from another molecule coordinated to copper. However, no single model can satisfy all the NMR data, including the relative distances. In model (1), proton 6 is not the closest to copper as indicated by the NMR results (Fig. 4); instead, the 2d-methyl group is the closest. In model (2), although proton 6 is closer to copper as indicated by [^{1}H]NMR data, the carboxyl group cannot be involved in the binding, which does not agree with the [^{13}C]NMR results. Model (3) is the combination of models (1) and (2). A proof for model (3) may not be possible with the fast-exchange limit for the NMR data. Since [^{13}C]NMR results also indicate that the imidazolinone ring may not directly participate in the binding, the copper complex of imazethapyr might contain copper coordinating to only the carboxyl group and pyridine ring nitrogen. A complex with copper coordinating to the pyridine ring nitrogen and the carboxyl group oxygen to form a chain of staggered four- or six-coordination complexes can be proposed for the imazethapyr complex, as proposed by Kleinstein and Webb[25] for metal complexes of nicotinic acid. Since similar metal interactions were observed for imazethapyr and CL303,135 compared to nicotinic and picolinic acid, respectively, it can be concluded that the structure of the imidazolinone complexes could be similar to those proposed for nicotinic acid and picolinic acids.[25] Although the imidazolinone ring in the imidazolinones appears to improve the binding, it does not seem to participate directly in the binding.

5 AHAS ENZYME ASSAY

As compared to CL303,135, imazethapyr is a slightly better inhibitor of wild-type AHAS isolated from BMS cells (Fig. 13). The I_{50} for imazethapyr is 4 μM, and 13 μM for CL303,135. Since the CL303,135 binds more strongly with copper than imazethapyr, it is interesting to determine the relative AHAS inhibition by the imidazolinones in the presence of copper. Cupric chloride alone at concentrations up to 25 μM did not inhibit AHAS. Therefore, a copper concentration less than 25 μM was used for studies of AHAS inhibition by imidazolinones. In the presence of copper, significantly higher concentrations of the imidazolinones were required to achieve the same degree of inhibition (Fig. 13). However, the effect of copper on the kinetics of inhibition was similar for both of the imidazolinones. This implies that differential interaction of copper with imidazolinones cannot be invoked to explain their different AHAS inhibition *in vitro*.

6 CONCLUSIONS

The binding studies of imazethapyr and CL303,135 with copper by UV & NMR and with europium by NMR clearly indicate that both of these imidazolinones form 2 : 1 imidazolinone : metal complexes, and that CL303,135 interacts more strongly with metals than imazethapyr. The strong affinity of CL303,135 for metals is due to the ability of CL303,135 to form chelate

bonds through the pyridine nitrogen and a carboxyl oxygen forming a stable five-membered ring. Imazethapyr cannot form such a chelate ring owing to steric factors caused by the relative positions of the carboxyl group and imidazolinone ring. The mechanisms of interaction of imazethapyr and CL303,135 are similar to those of their analogs nicotinic and picolinic acids, respectively. Although the imidazolinone ring in imazethapyr and CL303,135 does not participate directly in the binding, its presence does improve the binding affinity. Copper increases the I_{50} for AHAS enzyme for both of these imidazolinones. Although the different affinities of imazethapyr and CL303,135 for metals did not cause a significant difference in their AHAS inhibition *in vitro*, this may still be a factor that leads to differential uptake and translocation in plants.

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